GENOMIC SEQUENCE AND POLYPEPTIDES OF THE CIRCOVIRUS ASSOCIATED WITH PIGLET WEIGHT LOSS DISEASE (PWD), APPLICATIONS TO THE DIAGNOSIS, PREVENTION AND/OR TREATMENT OF THE INFECTION

The invention relates to the genomic sequence and nucleotide sequences coding for polypeptides of PWD circovirus, such as the structural and nonstructural polypeptides of said circovirus, as well as vectors including said sequences and cells or animals transformed by these vectors. The invention likewise relates to methods for detecting these nucleic acids or polypeptides and kits for diagnosing infection by the PWD circovirus. The invention is also directed at a method for selecting compounds capable of modulating the viral infection. The invention finally comprises pharmaceutical compositions, especially vaccines, for the prevention and/or the treatment of viral infections by PWD circovirus as well as the use of a vector according to the invention for the prevention and/or the treatment of diseases by gene therapy.

Piglet weight loss disease (PWD) or alternatively called fatal piglet wasting (FPW) has been widely described in North America (Harding, J.C., 1997), and authors have reported the existence of a relationship between this pathology and the presence of porcine circovirus (Daft, B. et al., 1996; Clark, E.G., 1997; Harding, J.C., 1997; Harding, J.C. and Clark, E.G., 1997; Nayar, G.P. et al., 1997). A porcine circovirus has already been demonstrated in established lines of cell cultures derived from pigs and chronically infected (Tischer, I., 1986, 1988, 1995; Dulac, G.C., 1989; Edwards, S., 1994; Allan, G.M., 1995 and McNeilly, F., 1996). This virus, during experimental infection of piglets, does not prove pathogenic for pigs (Tischer, I., 1986, Horner, G.W., 1991) and its nucleotide sequence has been determined and characterized (Tischer, I., 1982; Meehan, B.M. et

al., 1997; Mankertz., A., 1997). The porcine circovirus, called PCV virus, is part of the circovirus genus of the circoviridae family (Murphy, F.A. et al., 1995) whose virion has a circular DNA of size between 1.7 and 2.3 kb, which DNA comprises three open reading frames (ORF1 to ORF3), coding for a replication protein REP involved in the initiation and termination phase of rolling circular replication (RCR) (Heyraud-Nitschke, F., et al., 1995; Harding, M.R. et al., 1993; Hanson, S.F. et al., 1995; Fontes, E.P.B. et al., 1994), coding for a capsid protein (Boulton, L.H. et al., 1997; Hackland, A.F. et al., 1994; Chu, P.W.G. et al., 1993 and coding for a nonstructural protein called a dissemination protein (Lazarowitz., S.G. et al., 1989).

The authors of the present invention have noticed that the clinical signs perceptible in pigs and linked to infection by the PWD circovirus are very distinctive. These manifestations in general appear in pigs of 8 to 12 weeks of age, weaned for 4 to 8 weeks. The first signs are hypotonia without it being possible to speak of prostration. Rapidly (48 hours), the flanks hollow, the line of the spine becomes apparent, and the pigs "blanch". These signs are in general accompanied by hyperthermia, anorexia and most often by respiratory signs (coughing, dyspnea, polypnea). Transitory diarrhea can likewise appear. The disease state phase lasts approximately one month at the end of which the rate of mortality varies from 5 to 20%. To these mortalities, it is expedient to add a variable proportion (5-10%) of cadaveric animals which are no longer able to present an economic future. It is to be noted that outside of this critical stage of the end of post-weaning, no anomaly appears on the farms. In particular, the reproductive function is totally maintained.

On the epidemiological level, the first signs of this pathology appeared at the start of 1995 in the east of the Côtes d'Armor department in France, and the

farms affected are especially confined to this area of the department. In December 1996, the number of farms concerned could not be evaluated with precision because of the absence of a specific laboratory diagnostic method or of an epidemioligical surveillance system of the livestock. Based on the clinical facts as well as on results of postmortem examinations supplied by veterinarians, it is possible to estimate this number as several dozen (80-100). The contagiousness of the disease is weak to moderate. Cases are being reported outside the initial area and for the majority are following the transfer of animals coming from farms familiar with the problem. On the other hand, a characteristic of the condition is its strong remanence. Thus, farms which have been affected for a year are still affected in spite of the massive administration of therapeutics. Farms with clinical expression are drawn from various categories of specialization (breeders/fatteners, post-weaners/ fatteners) and different economic structures are concerned. In addition, the disorders appear even in farms where the rules of animal husbandry are respected.

Numerous postmortem examinations have been carried out either on farms or in the laboratory. The elements of the lesional table are disparate. The most constant macroscopic lesions are pneumonia which sometimes appears in patchy form as well as hypertrophy of the lymphatic ganglia. The other lesions above all affect the thoracic viscera including, especially, pericarditis and pleurisy. However, arthritis and gastric ulcers are also observed. The lesions revealed in the histological examination are essentially situated at the pulmonary level (interstitial pneumonia), ganglionic level (lymphoid depletion of the lymph nodes, giant cells) and renal level (glomerulonephritis, vasculitis). The infectious agents have been the subject of wide research. It has been

possible to exclude the intervention of pestiviruses and Aujeszky's disease. The disorders appear in the seropositive PDRS (Porcine Dysgenic and Respiratory Syndrome, an infection linked to an arteriovirus) herds, but it has not been possible to establish the role of the latter in the genesis of the disorders (the majority of the farms in Brittany are PDRS seropositive).

The authors of the present invention, with the aim of identifying the etiological agent responsible for PWD, have carried out "contact" tests between piglets which are obviously "ill" and SPF pigs (specific pathogen-free) from CNEVA (Centre National d'Etudes vétérinaires et Alimentaires, France). These tests allow the development of signs comparable to those observed on the farm to be observed in protected animal houses. The discrete signs such as moderate hyperthermia, anorexia and intermittent diarrhea appeared after one week of contact. It must be noted that the PDRS virus only diffused subsequent to the clinical signs. In addition, inocculations of organ homogenates of sick animals to healthy pigs allowed signs related to those observed on the farms to be reproduced, although with a lower incidence, linked to the favorable conditions of upkeep of the animals in the experimental installations.

Thus, the authors of the present invention have been able to demonstrate that the pathological signs appear as a well-defined entity affecting the pig at a particular stage of its growth.

This pathology has never been described in France. However, sparse information, especially Canadian, relates to similar facts.

The disorders cannot be mastered with the existing therapeutics.

The data collected both on the farm and by experimentation have allowed the following points to be higlighted:

- PWD is transmissible but its contagiousness is not very high,
- its etiological origin is of infectious and probably viral nature,
- PWD has a persistent character in the affected farms.

Considerable economic consequences ensue for the farms.

Thus, there is currently a significant need for a specific and sensitive diagnostic, whose production is practical and rapid, allowing the early detection of the infection.

A reliable, sensitive and practical test which allows the distinction between strains of porcine circovirus (PCV) is thus strongly desirable.

On the other hand, a need for efficient and well-tolerated treatment of infections with PWD circovirus likewise remains desirable, no vaccine currently being available against PWD circovirus.

Concerning PWD circovirus, it will probably be necessary to understand the role of the immune defense in the physiology and the pathology of the disease to develop satisfactory vaccines.

Fuller information concerning the biology of these strains, their interactions with their hosts, the associated infectivity phenomena and those of escape from the immune defenses of the host especially, and finally their implication in the development of associated pathologies, will allow a better understanding of these mechanisms. Taking into account the facts which have been mentioned above and which show in particular the limitations of combatting infection by the PWD circovirus, it is thus essential today on the one hand to develop molecular tools, especially starting from a better genetic knowledge of the PWD circovirus, but likewise to perfect novel preventive and therapeutic treatments, novel methods of diagnosis and specific, efficacious and tolerated novel

vaccine strategies. This is precisely the subject of the present invention.

The present invention relates to the nucleotide sequence of sequence SEQ ID No. 1, as represented in Figure 2, of the genome of PWD circovirus.

The nucleotide sequence of sequence SEQ ID No. 1 was deposited on 2 July 1997 at the GenBauk databank under the number nuc 1 AF012107.

The present invention likewise relates to nucleotide sequences, characterized in that they are selected from:

a) a nucleotide sequence of a specific fragment of the sequence SEQ ID No. 1;

b) a nucleotide sequence homologous to a nucleotide sequence such as defined in a);

c) a nucleotide sequence complementary to the sequence SEQ ID No. 1 or complementary to a nucleotide sequence such as defined in a) or b), and a nucleotide sequence of their corresponding RNA;

d) a nucleotide sequence capable of hybridizing under stringent conditions with a sequence such as defined in a), b) or c);

e) a nucleotide sequence comprising the sequence SEQ ID No. 1 or a sequence such as defined in a), b), c) or d); and

f) a nucleotide sequence modified by a nucleotide sequence such as defined in a), b), c), d) or e).

Nucleotide, polynucleotide or nucleic acid sequence will be understood according to the present invention as meaning both a double-stranded or single-stranded DNA in the monomeric and dimeric (so-called in tandem) forms and the transcription products of said DNAs.

It must be understood that the present invention does not relate to the genomic nucleotide sequences taken in their natural environment, that is to say in the natural state. It concerns sequences

which it has been possible to isolate, purify or partially purify, starting from separation methods such as, for example, ion-exchange chromatography, by exclusion based on molecular size, or by affinity, or alternatively fractionation techniques based on solubility in different solvents, or starting from methods of genetic engineering such as amplification, cloning and subcloning, it being possible for the sequences of the invention to be carried by vectors.

The nucleotide sequence SEQ ID No. 1 was obtained by sequencing of the genome by the Sanger method.

Specific fragment of a nucleotide sequence according to the invention will be understood as designating any nucleotide fragment of the PWD circovirus of length of at least 8 nucleotides, preferably at least 12 nucleotides, and even more preferentially at least 20 consecutive nucleotides of the sequence from which it originates, and having, after alignment and comparison with the corresponding fragments of known porcine circoviruses, at least one nucleotide or base of different nature. The specific nucleotide fragments of the PWD circovirus can easily be determined by referring to Figure 3 of the present invention in which the nucleotides or bases of the sequence SEQ ID No. 1 (circopordfp) are shown which are of different nature, after alignment of said sequence SEQ ID No. 1 with the other two sequences of known porcine circovirus (circopormeeh and circopormank).

Homologous nucleotide sequence in the sense of the present invention is understood as meaning a nucleotide sequence having at least one nucleotide sequence of a specific fragment, such as defined above, and a percentage identity with the bases of a nucleotide sequence according to the invention of at least 80%, preferably 90% or 95%, this percentage being purely statistical and it being possible to distribute the differences between the two nucleotide sequences at

random and over the whole of their length. Said "specific" homologous sequences can comprise, for example, the sequences corresponding to the genomic sequence or to the sequences of its fragments representative of variants of PWD circovirus. These specific homologous sequences can thus correspond to variations linked to mutations within the strain of PWD circovirus, and especially correspond to truncations, substitutions, deletions and/or additions of at least one nucleotide. Said homologous sequences can likewise correspond to variations linked to the degeneracy of the genetic code.

Complementary nucleotide sequence of a sequence of the invention is understood as meaning any DNA whose nucleotides are complementary to those of the sequence of the invention, and whose orientation is reversed (antiparallel sequence).

Hybridization under conditions of stringency with a nucleotide sequence according to the invention is understood as meaning a hybridization under conditions of temperature and ionic strength chosen in such a way that they allow the maintenance of the hybridization between two fragments of complementary DNA.

By way of illustration, conditions of great stringency of the hybridization step with the aim of defining the nucleotide fragments described above are advantageously the following.

The hybridization is carried out at a preferential temperature of 65°C in the presence of SSC buffer, 1 × SSC corresponding to 0.15 M NaCl and 0.05 M Na citrate. The washing steps, for example, can be the following:

- 2 × SSC, at ambient temperature followed by two washes with 2 × SSC, 0.5% SDS at 65°C; 2 × 0.5 [sic] × SSC, 0.5% SDS; at 65°C for 10 minutes each.

The conditions of intermediate stringency, using, for example, a temperature of 42°C in the presence of a 2 × SSC buffer, or of less stringency, for example a temperature of 37°C in the presence of a 2 × SSC buffer, respectively require a globally less significant complementarity for the hybridization between the two sequences.

The stringent hybridization conditions described above for a polynucleotide with a size of approximately 350 bases will be adapted by the person skilled in the art for oligonucleotides of greater or smaller size, according to the teaching of Sambrook et al., 1989.

Among the nucleotide sequences according to the invention, those are likewise preferred which can be used as a primer or probe in methods allowing the homologous sequences according to the invention to be obtained, these methods, such as the polymerase chain reaction (PCR), nucleic acid cloning and sequencing, being well known to the person skilled in the art.

Among said nucleotide sequences according to the invention, those are again preferred which can be used as a primer or probe in methods allowing the presence of PWD circovirus or one of its variants such as defined below to be diagnosed.

The nucleotide sequences according to the invention capable of modulating, of inhibiting or of inducing the gene expression of the PWD circovirus or one of its variants, and/or capable of modulating the replication cycle of PWD circovirus or one of its variants in the host cell and/or organism are likewise preferred. Replication cycle will be understood as designating the invasion and the multiplication of PWD circovirus or one of its variants, and its propagation from host cell to host cell in the host organism.

Among said nucleotide sequences according to the invention, those corresponding to open reading frames, called ORF sequences, and coding for

polypeptides, such as, for example, the sequences ORF1, ORF2 and ORF3 respectively corresponding to the nucleotide sequences between the positions 47 and 985 (ORF1), the positions 1022 and 1723 (ORF2) and the positions 38 and 658 (ORF3), the ends being included, the positions being determined with respect to the position of the nucleotides on the sequence SEQ ID No. 1 represented in Figure 2, are finally preferred.

The nucleotide sequence fragments according to the invention can be obtained, for example, by specific amplification, such as PCR, or after digestion with appropriate restriction enzymes of nucleotide sequences according to the invention, these methods in particular being described in the work of Sambrook et al., 1989. Said representative fragments can likewise be obtained by chemical synthesis when their size is not very large and according to methods well known to persons skilled in the art.

Modified nucleotide sequence will be understood as meaning any nucleotide sequence obtained by mutagenesis according to techniques well known to the person skilled in the art, and containing modifications with respect to the normal sequences according to the invention, for example mutations in the regulatory and/or promoter sequences of polypeptide expression, especially leading to a modification of the rate of expression of said polypeptide or to a modulation of the replicative cycle.

Modified nucleotide sequence will likewise be understood as meaning any nucleotide sequence coding for a modified polypeptide such as defined below.

The present invention relates to nucleotide sequences of PWD circovirus according to the invention, characterized in that they are selected from the sequences ORF1, ORF2 and ORF3 and such as defined above.

The invention likewise relates to nucleotide sequences characterized in that they comprise a nucleotide sequence selected from:

a) a nucleotide sequence ORF1 to ORF3 according to the invention;

b) a nucleotide sequence of a specific fragment of a sequence ORF1 to ORF3 according to the invention or of a sequence such as defined in a);

c) a homologous nucleotide sequence having at least 80% identity with a nucleotide sequence ORF1 to ORF3 according to the invention or such as defined in a) or b);

d) a complementary nucleotide sequence or sequence of RNA corresponding to a sequence ORF1 to ORF3 according to the invention or such as defined in a), b) or c); and

e) a nucleotide sequence modified by a sequence ORF1 to ORF3 according to the invention or such as defined in a), b), c) or d).

As far as homology with the nucleotide sequences ORF1 to ORF3 is concerned, the specific homologous sequences such as defined above having a percentage identity with the bases of one of the nucleotide sequences ORF1 to ORF3 of at least 80%, preferably 90% or 95%, are preferred. Said specific homologous sequences can comprise, for example, the sequences corresponding to the sequences ORF1 to ORF3 of PWD circovirus variant. In the same manner, these specific homologous sequences can correspond to variations linked to mutations within the strain of PWD circovirus and especially correspond to truncations, substitutions, deletions and/or additions of at least one nucleotide.

Preferably, the invention relates to the nucleotide sequences according to the invention, characterized in that they comprise a nucleotide sequence selected from the following sequences:

a) 170 5' TGTGGCGA 3';

b) 450 5' AGTTTCCT 3';
c) 1026 5' TCATTTAGAGGGTCTTTCAG 3';
d) 1074 5' GTCAACCT 3';
e) 1101 5' GTGGTTGC 3';
f) 1123 5' AGCCCAGG 3';
g) 1192 5' TTGGCTGG 3';
h) 1218 5' TCTAGCTCTGGT 3';
i) 1501 5' ATCTCAGCTCGT 3';
j) 1536 5' TGTCCTCCTCTT 3';
k) 1563 5' TCTCTAGA 3';
l) 1623 5' TGTACCAA 3';
m) 1686 5' TCCGTCTT 3';

and their complementary sequence.

In the list of nucleotide sequences a)-m) above, the underlined nucleotides are mutated with respect to the two known sequences of circovirus which are nonpathogenic to pigs. The number preceding the nucleotide sequence represents the position of the first nucleotide of said sequence in the sequence SEQ ID No. 1.

The invention comprises the polypeptides encoded by a nucleotide sequence according to the invention, preferably a polypeptide whose sequence is represented by a specific fragment of one of the six sequences of amino acids represented in Figure 3, these six amino acid sequences corresponding to the polypeptides which can be encoded according to one of the three possible reading frames of the sequence SEQ ID No. 1 or of the DNA sequence of its complementary strand.

The invention likewise relates to the polypeptides, characterized in that they comprise a polypeptide selected from the amino acid sequences SEQ ID No. 2, SEQ ID No. 3 and SEQ ID No. 4, represented in Figures 4, 5 and 6, respectively.

The invention also relates to the polypeptides, characterized in that they comprise a polypeptide selected from:

a) a polypeptide according to the invention;

b) a specific fragment of at least 5 amino acids of a polypeptide according to the invention and such as defined in a);

c) a polypeptide homologous to a polypeptide according to the invention, or such as defined in a) or b);

d) a specific biologically active fragment of a polypeptide according to the invention, or such as defined in a), b) or c); and

e) a polypeptide modified by a polypeptide according to the invention, or such as defined in a), b), c) or d).

In the present description, the terms polypeptide, peptide and protein are interchangeable.

It must be understood that the invention does not relate to the polypeptides in natural form, that is to say that they are not taken in their natural environment but that they can be isolated or obtained by purification from natural sources, or else obtained by genetic recombination, or alternatively by chemical synthesis and that they can thus contain unnatural amino acids, as will be described below.

Polypeptide fragment according to the invention is understood as designating a polypeptide containing at least 5 amino acids, preferably 10 amino acids and [sic] 15 amino acids.

In the present invention, specific polypeptide fragment is understood as designating the polypeptide fragment encoded by a specific fragment nucleotide sequence according to the invention.

Homologous polypeptide will be understood as designating the polypeptides having a specific fragment of polypeptide according to the invention, and having, with respect to the natural polypeptide, certain modifications such as, in particular, a deletion, addition or substitution of at least one amino acid, a truncation, a prolongation, a chimeric fusion, and/or a

mutation. Among the "specific" homologous polypeptides, those are preferred whose amino acid sequence has at least 80%, preferably 90%, homology with the sequences of amino acids of polypeptides according to the invention.

In the case of a substitution, one or more consecutive or nonconsecutive amino acids are replaced by "equivalent" amino acids. The expression "equivalent" amino acid is directed here at designating any amino acid capable of being substituted by one of the amino acids of the base structure without, however, essentially modifying the biological activities of the corresponding peptides and such that they will be defined by the following.

These equivalent amino acids can be determined either by depending on their structural homology with the amino acids which they substitute, or on results of comparative tests of biological activity between the different polypeptides, which are capable of being carried out.

By way of example, the possibilities of substitutions capable of being carried out without resulting in an extensive modification of the biological activity of the corresponding modified polypeptides will be mentioned, the replacement, for example, of leucine by valine or isoleucine, of aspartic acid by glutamic acid, of glutamine by asparagine, of arginine by lysine etc., the reverse substitutions naturally being envisageable under the same conditions.

The specific homologous polypeptides likewise correspond to polypeptides encoded by the specific homologous nucleotide sequences such as defined above and thus comprise in the present definition the polypeptides which are mutated or correspond to variants which can exist in PWD circovirus, and which especially correspond to truncations, substitutions,

deletions and/or additions of at least one amino acid residue.

Specific biologically active fragment of a polypeptide according to the invention will be understood in particular as designating a specific polypeptide fragment, such as defined above, having at least one of the characteristics of polypeptides according to the invention, especially in that it is:

- capable of inducing an immunogenic reaction directed against the PWD circovirus; and/or
- capable of being recognized by a specific antibody of a polypeptide according to the invention; and/or
- capable of linking to a polypeptide or to a nucleotide sequence of PWD circovirus; and/or
- capable of exerting a physiological activity, even partial, such as, for example, a dissemination or structural (capsid) activity; and/or
- capable of modulating, of inducing or of inhibiting the expression of PWD circovirus gene or one of its variants, and/or capable of modulating the replication cycle of PWD circovirus or one of its variants in the cell and/or the host organism.

The polypeptide fragments according to the invention can correspond to isolated or purified fragments naturally present in the PWD circovirus or correspond to fragments which can be obtained by cleavage of said polypeptide by a proteolytic enzyme, such as trypsin or chymotrypsin or collagenase, or by a chemical reagent, such as cyanogen bromide (CNBr) or alternatively by placing said polypeptide in a very acidic environment, for example at pH 2.5. Such polypeptide fragments can likewise just as easily be prepared by chemical synthesis, from hosts transformed by an expression vector according to the invention containing a nucleic acid allowing the expression of

said fragments, placed under the control of appropriate regulation and/or expression elements.

"Modified polypeptide" of a polypeptide according to the invention is understood as designating a polypeptide obtained by genetic recombination or by chemical synthesis as will be described below, having at least one modification with respect to the normal sequence. These modifications will especially be able to bear on amino acids at the origin of a specificity, of pathogenicity and/or of virulence, or at the origin of the structural conformation, and of the capacity of membrane insertion of the polypeptide according to the invention. It will thus be possible to create polypeptides of equivalent, increased or decreased activity, and of equivalent, narrower, or wider specificity. Among the modified polypeptides, it is necessary to mention the polypeptides in which up to 5 amino acids can be modified, truncated at the N- or C-terminal end, or even deleted or added.

As is indicated, the modifications of the polypeptide will especially have as objective:

- to render it capable of modulating, of inhibiting or of inducing the gene expression of PWD circovirus and/or one of its variants and/or capable of modulating the replication cycle of PWD circovirus and/or one of its variants in the cell and/or the host organism,
- of allowing its incorporation into vaccine compositions,
- of modifying its bioavailability as a compound for therapeutic use.

The methods allowing said modulations on eukaryotic or prokaryotic cells to be demonstrated are well known to the person skilled in the art. It is likewise well understood that it will be possible to use the nucleotide sequences coding for said modified polypeptides for said modulations, for example through vectors according to the invention and described below,

in order, for example, to prevent or to treat the pathologies linked to the infection.

The preceding modified polypeptides can be obtained by using combinatorial chemistry, in which it is possible to systematically vary parts of the polypeptide before testing them on models, cell cultures or microorganisms for example, to select the compounds which are most active or have the properties sought.

Chemical synthesis likewise has the advantage of being able to use:

- unnatural amino acids, or
- nonpeptide bonds.

Thus, in order to improve the duration of life of the polypeptides according to the invention, it may be of interest to use unnatural amino acids, for example in D form, or else amino acid analogs, especially sulfur-containing forms, for example.

Finally, it will be possible to integrate the structure of the polypeptides according to the invention, its specific or modified homologous forms, into chemical structures of polypeptide type or others. Thus, it may be of interest to provide at the N- and C-terminal ends compounds not recognized by the proteases.

The nucleotide sequences coding for a polypeptide according to the invention are likewise part of the invention.

The invention likewise relates to nucleotide sequences utilizable as a primer or probe, characterized in that said sequences are selected from the nucleotide sequences according to the invention.

Among the pairs of nucleotide sequences utilizable as a pair of primers according to the invention, the pairs of primers selected from the following pairs are preferred:

a) 5' GTG TGC TCG ACA TTG GTG TG 3', and
   5' TGG AAT GTT AAC GAG CTG AG 3';

b) 5' GTG TGC TCG ACA TTG GTG TG 3', and
5' CTC GCA GCC ATC TTG GAA TG 3';
c) 5' CGC GCG TAA TAC GAC TCA CT 3', and
5' GTG TGC TCG ACA TTG GTG TG 3';
d) 5' CGC GCG TAA TAC GAC TCA CT 3', and
5' CTC GCA GCC ATC TTG GAA TG 3'.

The cloning and the sequencing of the PWD circovirus has allowed it to be identified, after comparative analysis with the nucleotide sequences of other porcine circoviruses, that, among the sequences of fragments of these nucleic acids, were those which are strictly specific to the PWD circovirus and those which correspond to a consensus sequence of other porcine circoviruses of cell lines.

There is likewise a great need for nucleotide sequences utilizable as a primer or probe specific to the whole of the other known and nonpathogenic porcine circoviruses.

Said consensus nucleotide sequences specific to all circoviruses, other than PWD circovirus, are easily identifiable in Figure 3, and are part of the invention.

Among said consensus nucleotide sequences, that which is characterized in that it is part of the following pair of primers is preferred:

a) 5' GTG TGC TCG ACA TTG GTG TG 3', and
5' TGG AAT GTT AAC TAC CTC AA 3'.

It is well understood that the present invention likewise relates to specific polypeptides of known porcine circoviruses other than PWD circovirus, encoded by said consensus nucleotide sequences, capable of being obtained by purification from natural polypeptides, by genetic recombination or by chemical synthesis by procedures well known to the person skilled in the art and such as described in particular below. In the same manner, the labeled or unlabeled mono- or polyclonal antibodies directed against said

specific polypeptides encoded by said consensus nucleotide sequences are also part of the invention.

It will be possible to use said consensus nucleotide sequences, said corresponding polypeptides as well as said antibodies directed against said polypeptides in procedures or sets for detection and/or identification such as described below, in place of or in addition to nucleotide sequences, polypeptides or antibodies according to the invention, specific to the PWD circovirus.

These protocols have been improved for the differential detection of the circular monomeric forms of specific replicative forms of the virion or of the DNA in replication and the dimeric forms found in so-called in-tandem molecular constructs.

The invention additionally relates to the use of a nucleotide sequence according to the invention as a primer or probe for the detection and/or the amplification of nucleic acid sequences.

The nucleotide sequences according to the invention can thus be used to amplify nucleotide sequences, especially by the PCR technique (polymerase chain reaction) (Erlich, 1989; Innis et al., 1990; Rolfs et al., 1991; and White et al., 1997).

These oligodeoxyribonucleotide or oligoribonucleotide primers advantageously have a length of at least 8 nucleotides, preferably of at least 12 nucleotides, and even more preferentially at least 20 nucleotides.

Other amplification techniques of the target nucleic acid can be advantageously employed as alternatives to PCR.

The nucleotide sequences of the invention, in particular the primers according to the invention, can likewise be employed in other procedures of amplification of a target nucleic acid, such as:

- the TAS technique (Transcription-based Amplification System), described by Kwoh et al. in 1989;
- the 3SR technique (Self-Sustained Sequence Replication), described by Guatelli et al. in 1990;
- the NASBA technique (Nucleic Acid Sequence Based Amplification), described by Kievitis et al. in 1991;
- the SDA technique (Strand Displacement Amplification) (Walker et al., 1992);
- the TMA technique (Transcription Mediated Amplification).

The polynucleotides of the invention can also be employed in techniques of amplification or of modification of the nucleic acid serving as a probe, such as:

- the LCR technique (Ligase Chain Reaction), described by Landegren et al. in 1988 and improved by Barany et al. in 1991, which employs a thermostable ligase;
- the RCR technique (Repair Chain Reaction), described by Segev in 1992;
- the CPR technique (Cycling Probe Reaction), described by Duck et al. in 1990;
- the amplification technique with Q-beta replicase, described by Miele et al. in 1983 and especially improved by Chu et al. in 1986, Lizardi et al. in 1988, then by Burg et al. as well as by Stone et al. in 1996.

In the case where the target polynucleotide to be detected is possibly an RNA, for example an mRNA, it will be possible to use, prior to the employment of an amplification reaction with the aid of at least one primer according to the invention or to the employment of a detection procedure with the aid of at least one probe of the invention, an enzyme of reverse transcriptase type in order to obtain a cDNA from the

RNA contained in the biological sample. The cDNA obtained will thus serve as a target for the primer(s) or the probe(s) employed in the amplification or detection procedure according to the invention.

The detection probe will be chosen in such a manner that it hybridizes with the target sequence or the amplicon generated from the target sequence. By way of sequence, such a probe will advantageously have a sequence of at least 12 nucleotides, in particular of at least 20 nucleotides, and preferably of at least 100 nucleotides.

The invention also comprises the nucleotide sequences utilizable as a probe or primer according to the invention, characterized in that they are labeled with a radioactive compound or with a nonradioactive compound.

The unlabeled nucleotide sequences can be used directly as probes or primers, although the sequences are generally labeled with a radioactive element (^{32}P, ^{35}S, ^{3}H, ^{125}I) or with a nonradioactive molecule (biotin, acetylaminofluorene, digoxigenin, 5-bromodeoxyuridine, fluorescein) to obtain probes which are utilizable for numerous applications.

Examples of nonradioactive labeling of nucleotide sequences are described, for example, in French Patent No. 78.10975 or by Urdea et al. or by Sanchez-Pescador et al. in 1988.

In the latter case, it will also be possible to use one of the labeling methods described in patents FR-2 422 956 and FR-2 518 755.

The hybridization technique can be carried out in various manners (Matthews et al., 1988). The most general method consists in immobilizing the nucleic acid extract of cells on a support (such as nitrocellulose, nylon, polystyrene) and in incubating, under well-defined conditions, the immobilized target nucleic acid with the probe. After hybridization, the excess of probe is eliminated and the hybrid molecules

formed are detected by the appropriate method (measurement of the radioactivity, of the fluorescence or of the enzymatic activity linked to the probe).

The invention likewise comprises the nucleotide sequences according to the invention, characterized in that they are immobilized on a support, covalently or noncovalently.

According to another advantageous mode of employing nucleotide sequences according to the invention, the latter can be used immobilized on a support and can thus serve to capture, by specific hybridization, the target nucleic acid obtained from the biological sample to be tested. If necessary, the solid support is separated from the sample and the hybridization complex formed between said capture probe and the target nucleic acid is then detected with the aid of a second probe, a so-called detection probe, labeled with an easily detectable element.

Another subject of the present invention is a vector for the cloning and/or expression of a sequence, characterized in that it contains a nucleotide sequence according to the invention.

The vectors according to the invention, characterized in that they contain the elements allowing the expression and/or the secretion of said nucleotide sequences in a determined host cell, are likewise part of the invention.

The vector must then contain a promoter, signals of initiation and termination of translation, as well as appropriate regions of regulation of transcription. It must be able to be maintained stably in the host cell and can optionally have particular signals specifying the secretion of the translated protein. These different elements are chosen as a function of the host cell used. To this end, the nucleotide sequences according to the invention can be inserted into autonomous replication vectors within the chosen host, or integrated vectors of the chosen host.

Such vectors will be prepared according to the methods currently used by the person skilled in the art, and it will be possible to introduce the clones resulting therefrom into an appropriate host by standard methods, such as, for example, lipofection, electroporation and thermal shock.

The vectors according to the invention are, for example, vectors of plasmid or viral origin.

A preferred vector for the expression of polypeptides of the invention is baculovirus.

The vector pBS KS in which is inserted the in-tandem DNA sequence of the PWD circovirus (or DFP) as deposited at the CNCM on 3 July 1997, under the number I-1891, is likewise preferred.

These vectors are useful for transforming host cells in order to clone or to express the nucleotide sequences of the invention.

The invention likewise comprises the host cells transformed by a vector according to the invention.

These cells can be obtained by the introduction into host cells of a nucleotide sequence inserted into a vector such as defined above, then the culturing of said cells under conditions allowing the replication and/or expression of the transfected nucleotide sequence.

The host cell can be selected from prokaryotic or eukaryotic systems, such as, for example, bacterial cells (Olins and Lee, 1993), but likewise yeast cells (Buckholz, 1993), as well as animal cells, in particular the cultures of mammalian cells (Edwards and Aruffo, 1993), and especially Chinese hamster ovary (CHO) cells, but likewise the cells of insects in which it is possible to use procedures employing baculoviruses, for example (Luckow, 1993).

A preferred host cell for the expression of the proteins of the invention is constituted by sf9 insect cells.

A more preferred host cell according to the invention is E. coli, such as deposited at the CNCM on 3 July 1997, under the number I-1891.

The invention likewise relates to animals comprising one of said transformed cells according to the invention.

The obtainment of transgenic animals according to the invention overexpressing one or more of the genes of PWD circovirus or part of the genes will be preferably carried out in rats, mice or rabbits according to methods well known to the person skilled in the art, such as by viral or nonviral transfections. It will be possible to obtain the transgenic animals overexpressing one or more of said genes by transfection of multiple copies of said genes under the control of a strong promoter of ubiquitous nature, or selective for one type of tissue. It will likewise be possible to obtain the transgenic animals by homologous recombination in embryonic cell strains, transfer of these cell strains to embryos, selection of the affected chimeras at the level of the reproductive lines, and growth of said chimeras.

The transformed cells as well as the transgenic animals according to the invention are utilizable in procedures for preparation of recombinant polypeptides.

It is today possible to produce recombinant polypeptides in relatively large quantity by genetic engineering using the cells transformed by expression vectors according to the invention or using transgenic animals according to the invention.

The procedures for preparation of a polypeptide of the invention in recombinant form, characterized in that they employ a vector and/or a cell transformed by a vector according to the invention and/or a transgenic animal comprising one of said transformed cells according to the invention, are themselves comprised in the present invention.

Among said procedures for preparation of a polypeptide of the invention in recombinant form, the preparation procedures employing a vector, and/or a cell transformed by said vector and/or a transgenic animal comprising one of said transformed cells, containing a nucleotide sequence according to the invention coding for a polypeptide of PWD circovirus, are preferred.

The recombinant polypeptides obtained as indicated above can just as well be present in glycosylated form as in nonglycosylated form and can or cannot have the natural tertiary structure.

A preferred variant consists in producing a recombinant polypeptide fused to a "carrier" protein (chimeric protein). The advantage of this system is that it allows a stabilization of and a decrease in the proteolysis of the recombinant product, an increase in the solubility in the course of renaturation in vitro and/or a simplification of the purification when the fusion partner has an affinity for a specific ligand.

More particularly, the invention relates to a procedure for preparation of a polypeptide of the invention comprising the following steps:

a) culture of transformed cells under conditions allowing the expression of a recombinant polypeptide of nucleotide sequence according to the invention;

b) if need be, recovery of said recombinant polypeptide.

When the procedure for preparation of a polypeptide of the invention employs a transgenic animal according to the invention, the recombinant polypeptide is then extracted from said animal.

The invention also relates to a polypeptide which is capable of being obtained by a procedure of the invention such as described previously.

The invention also comprises a procedure for preparation of a synthetic polypeptide, characterized

in that it uses a sequence of amino acids of polypeptides according to the invention.

The invention likewise relates to a synthetic polypeptide obtained by a procedure according to the invention.

The polypeptides according to the invention can likewise be prepared by techniques which are conventional in the field of the synthesis of peptides. This synthesis can be carried out in homogeneous solution or in solid phase.

For example, recourse can be made to the technique of synthesis in homogeneous solution described by Houben-Weyl in 1974.

This method of synthesis consists in successively condensing, two by two, the successive amino acids in the order required, or in condensing amino acids and fragments formed previously and already containing several amino acids in the appropriate order, or alternatively several fragments previously prepared in this way, it being understood that it will be necessary to protect beforehand all the reactive functions carried by these amino acids or fragments, with the exception of amine functions of one and carboxyls of the other or vice-versa, which must normally be involved in the formation of peptide bonds, especially after activation of the carboxyl function, according to the methods well known in the synthesis of peptides.

According to another preferred technique of the invention, recourse will be made to the technique described by Merrifield.

To make a peptide chain according to the Merrifield procedure, recourse is made to a very porous polymeric resin, on which is immobilized the first C-terminal amino acid of the chain. This amino acid is immobilized on a resin through its carboxyl group and its amine function is protected. The amino acids which are going to form the peptide chain are thus

immobilized, one after the other, on the amino group, which is deprotected beforehand each time, of the portion of the peptide chain already formed, and which is attached to the resin. When the whole of the desired peptide chain has been formed, the protective groups of the different amino acids forming the peptide chain are eliminated and the peptide is detached from the resin with the aid of an acid.

The invention additionally relates to hybrid polypeptides having at least one polypeptide according to the invention, and a sequence of a polypeptide capable of inducing an immune response in man or animals.

Advantageously, the antigenic determinant is such that it is capable of inducing a humoral and/or cellular response.

It will be possible for such a determinant to comprise a polypeptide according to the invention in glycosylated form used with a view to obtaining immunogenic compositions capable of inducing the synthesis of antibodies directed against multiple epitopes. Said polypeptides or their glycosylated fragments are likewise part of the invention.

These hybrid molecules can be formed, in part, of a polypeptide carrier molecule or of fragments thereof according to the invention, associated with a possibly immunogenic part, in particular an epitope of the diphtheria toxin, the tetanus toxin, a surface antigen of the hepatitis B virus (patent FR 79 21811), the VP1 antigen of the poliomyelitis virus or any other viral or bacterial toxin or antigen.

The procedures for synthesis of hybrid molecules encompass the methods used in genetic engineering for constructing hybrid nucleotide sequences coding for the polypeptide sequences sought. It will be possible, for example, to refer advantageously to the technique for obtainment of genes coding for fusion proteins described by Minton in 1984.

Said hybrid nucleotide sequences coding for a hybrid polypeptide as well as the hybrid polypeptides according to the invention characterized in that they are recombinant polypeptides obtained by the expression of said hybrid nucleotide sequences are likewise part of the invention.

The invention likewise comprises the vectors characterized in that they contain one of said hybrid nucleotide sequences. The host cells transformed by said vectors, the transgenic animals comprising one of said transformed cells as well as the procedures for preparation of recombinant polypeptides using said vectors, said transformed cells and/or said transgenic animals are, of course, likewise part of the invention.

The polypeptides according to the invention, the antibodies according to the invention described below and the nucleotide sequences according to the invention can advantageously be employed *in vitro* and/or *in vivo* in procedures for the detection and/or identification of PWD circovirus, or of porcine circovirus other than the PWD circovirus, in a biological sample (biological tissue or fluid) capable of containing them. These procedures, according to the specificity of the polypeptides, the antibodies and the nucleotide sequences according to the invention which will be used, will in particular be able to detect and/or to identify the PWD circovirus or the porcine circovirus other than the PWD circovirus.

The polypeptides according to the invention can advantageously be employed in a procedure for the detection and/or the identification of PWD circovirus or porcine circovirus other than the PWD circovirus, in a biological sample (biological tissue or fluid) capable of containing them, characterized in that it comprises the following steps:

a) contacting of this biological sample with a polypeptide or one of its fragments according to the invention (under conditions allowing an

immunological reaction between said polypeptide and the antibodies possibly present in the biological sample);

b) demonstration of the antigen-antibody complexes possibly formed.

Preferably, the biological sample is formed by a fluid, for example a pig serum, whole blood or biopsies.

Any conventional procedure can be employed for carrying out such a detection of the antigen-antibody complexes possibly formed.

By way of example, a preferred method brings into play immunoenzymatic processes according to the ELISA technique, by immunofluorescence, or radioimmunological processes (RIA) or their equivalent.

Thus, the invention likewise relates to the polypeptides according to the invention, labeled with the aid of an adequate label such as of the enzymatic, fluorescent or radioactive type.

Such methods comprise, for example, the following steps:

- deposition of determined quantities of a polypeptide composition according to the invention in the wells of a microtiter plate,
- introduction into said wells of increasing dilutions of serum, or of a biological sample other than that defined previously, having to be analyzed,
- incubation of the microplate,
- introduction into the wells of the microtiter plate of labeled antibodies directed against pig immunoglobulins, the labeling of these antibodies having been carried out with the aid of an enzyme selected from those which are capable of hydrolyzing a substrate by modifying the absorption of the radiation of the latter, at least at a determined wavelength, for example at 550 nm,

- detection, by comparison with a control test, of the quantity of hydrolyzed substrate.

The invention likewise relates to a kit or set for the detection and/or identification of PWD circovirus or of porcine circovirus other than the PWD circovirus, characterized in that it comprises the following elements:

- a polypeptide according to the invention,
- if need be, the reagents for the formation of the medium favorable to the immunological or specific reaction,
- the reagents allowing the detection of the antigen-antibody complexes produced by the immunological reaction between the polypeptide(s) of the invention and the antibodies possibly present in the biological sample, these reagents likewise being able to carry a label, or to be recognized in their turn by a labeled reagent, more particularly in the case where the polypeptide according to the invention is not labeled,
- if need be, a biological reference sample (negative control) devoid of antibodies recognized by a polypeptide according to the invention,
- if need be, a biological reference sample (positive control) containing a predetermined quantity of antibodies recognized by a polypeptide according to the invention.

The polypeptides according to the invention allow monoclonal or polyclonal antibodies to be prepared which are characterized in that they specifically recognize the polypeptides according to the invention. It will advantageously be possible to prepare the monoclonal antibodies from hybridomas according to the technique described by Kohler and Milstein in 1975. It will be possible to prepare the polyclonal antibodies, for example, by immunization of an animal, in particular a mouse, with a polypeptide or a DNA, according to the invention, associated with an

adjuvant of the immune response, and then purification of the specific antibodies contained in the serum of the immunized animals on an affinity column on which the polypeptide which has served as an antigen has previously been immobilized. The polyclonal antibodies according to the invention can also be prepared by purification, on an affinity column on which a polypeptide according to the invention has previously been immobilized, of the antibodies contained in the serum of pigs infected by the PWD circovirus.

The invention likewise relates to mono- or polyclonal antibodies or their fragments, or chimeric antibodies, characterized in that they are capable of specifically recognizing a polypeptide according to the invention.

It will likewise be possible for the antibodies of the invention to be labeled in the same manner as described previously for the nucleic probes of the invention, such as a labeling of enzymatic, fluorescent or radioactive type.

The invention is additionally directed at a procedure for the detection and/or identification of PWD circovirus or of porcine circovirus other than the PWD circovirus, in a biological sample, characterized in that it comprises the following steps:

a) contacting of the biological sample (biological tissue or fluid) with a mono- or polyclonal antibody according to the invention (under conditions allowing an immunological reaction between said antibodies and the polypeptides of the PWD circovirus or of porcine circovirus other than the PWD circovirus, possibly present in the biological sample);

b) demonstration of the antigen-antibody complex possibly formed.

Likewise within the scope of the invention is a kit or set for the detection and/or the identification of the PWD circovirus or of porcine circovirus other

than the PWD circovirus, characterized in that it comprises the following components:

- a polyclonal or monoclonal antibody according to the invention, if need be labeled;
- if need be, a reagent for the formation of the medium favorable to the carrying out of the immunological reaction;
- a reagent allowing the detection of the antigen-antibody complexes produced by the immunological reaction, this reagent likewise being able to carry a label, or being capable of being recognized in its turn by a labeled reagent, more particularly in the case where said monoclonal or polyclonal antibody is not labeled;
- if need be, reagents for carrying out the lysis of cells of the sample tested.

The present invention likewise relates to a procedure for the detection and/or the identification of the PWD circovirus or of porcine circovirus other than the PWD circovirus, in a biological sample, characterized in that it employs a nucleotide sequence according to the invention.

More particularly, the invention relates to a procedure for the detection and/or the identification of the PWD circovirus or of porcine circovirus other than the PWD circovirus, in a biological sample, characterized in that it contains the following steps:

a) if need be, isolation of the DNA from the biological sample to be analyzed;

b) specific amplification of the DNA of the sample with the aid of at least one primer, or a pair of primers, according to the invention;

c) demonstration of the amplification products.

These can be detected, for example, by the technique of molecular hybridization utilizing a nucleic probe according to the invention. This probe will advantageously be labeled with a nonradioactive (cold probe) or radioactive element.

For the purposes of the present invention, "DNA of the biological sample" or "DNA contained in the biological sample" will be understood as meaning either the DNA present in the biological sample considered, or possibly the cDNA obtained after the action of an enzyme of reverse transcriptase type on the RNA present in said biological sample.

Another aim of the present invention consists in a procedure according to the invention, characterized in that it comprises the following steps:

a) contacting of a nucleotide probe according to the invention with a biological sample, the DNA contained in the biological sample having, if need be, previously been made accessible to hybridization under conditions allowing the hybridization of the probe with the DNA of the sample;

b) demonstration of the hybrid formed between the nucleotide probe and the DNA of the biological sample.

The present invention also relates to a procedure according to the invention, characterized in that it comprises the following steps:

a) contacting of a nucleotide probe immobilized on a support according to the invention with a biological sample, the DNA of the sample having, if need be, previously been made accessible to hybridization, under conditions allowing the hybridization of the probe with the DNA of the sample;

b) contacting of the hybrid formed between the nucleotide probe immobilized on a support and the DNA contained in the biological sample, if need be after elimination of the DNA of the biological sample which has not hybridized with the probe, with a nucleotide probe labeled according to the invention;

c) demonstration of the novel hybrid formed in step b).

According to an advantageous embodiment of the procedure for detection and/or identification defined previously, this is characterized in that, prior to

step a), the DNA of the biological sample is first amplified with the aid of at least one primer according to the invention.

The invention is additionally directed at a kit or set for the detection and/or the identification of the PWD circovirus or of porcine circovirus other than the PWD circovirus, characterized in that it comprises the following elements:

a) a nucleotide probe according to the invention;

b) if need be, the reagents necessary for the carrying out of a hybridization reaction;

c) if need be, at least one primer according to the invention as well as the reagents necessary for an amplification reaction of the DNA.

The invention likewise relates to a kit or set for the detection and/or the identification of the PWD circovirus or of porcine circovirus other than the PWD circovirus, characterized in that it comprises the following components:

a) a nucleotide probe, called a capture probe, according to the invention;

b) an oligonucleotide probe, called a revealing probe, according to the invention,

c) if need be, at least one primer according to the invention, as well as the reagents necessary for an amplification reaction of the DNA.

The invention also relates to a kit or set for the detection and/or identification of the PWD circovirus or of porcine circovirus other than the PWD circovirus, characterized in that it comprises the following elements:

a) at least one primer according to the invention;

b) if need be, the reagents necessary for carrying out a DNA amplification reaction;

c) if need be, a component allowing the sequence of the amplified fragment to be verified, more particularly an oligonucleotide probe according to the invention.

The invention additionally relates to the use of a nucleotide sequence according to the invention, of a polypeptide according to the invention, of an antibody according to the invention, of a cell according to the invention, and/or of an animal transformed according to the invention, for the selection of an organic or inorganic compound capable of modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of PWD circovirus or capable of inducing or of inhibiting the pathologies linked to an infection by the PWD circovirus.

The invention likewise comprises a method of selection of compounds capable of binding to a polypeptide or one of its fragments according to the invention, capable of binding to a nucleotide sequence according to the invention, or capable of recognizing an antibody according to the invention, and/or capable of modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of PWD circovirus or capable of inducing or inhibiting the pathologies linked to an infection by the PWD circovirus, characterized in that it comprises the following steps:

a) contacting of said compound with said polypeptide, said nucleotide sequence, or with a cell transformed according to the invention and/or administration of said compound to an animal transformed according to the invention;

b) determination of the capacity of said compound to bind to said polypeptide or said nucleotide sequence, or to modulate, induce or inhibit the expression of genes, or to modulate the growth or the replication or [sic] of the PWD circovirus, or to induce or inhibit in said transformed animal the pathologies linked to an infection by PWD circovirus (designated activity of said compound).

The compounds capable of being selected can be organic compounds such as polypeptides or carbohydrates or any other organic or inorganic compounds already known, or novel organic compounds elaborated by molecular modelling techniques and obtained by chemical or biochemical synthesis, these techniques being known to the person skilled in the art.

It will be possible to use said selected compounds to modulate the cellular replication of the PWD circovirus and thus to control infection by this virus, the methods allowing said modulations to be determined being well known to the person skilled in the art.

This modulation can be carried out, for example, by an agent capable of binding to a protein and thus of inhibiting or of potentiating its biological activity, or capable of binding to an envelope protein of the external surface of said virus and of blocking the penetration of said virus into the host cell or of favoring the action of the immune system of the infected organism directed against said virus. This modulation can likewise be carried out by an agent capable of binding to a nucleotide sequence of a DNA of said virus and of blocking, for example, the expression of a polypeptide whose biological or structural activity is necessary for the replication or for the proliferation of said virus host cells to host cells in the host animal.

The invention relates to the compounds capable of being selected by a selection method according to the invention.

The invention likewise relates to a pharmaceutical composition comprising a compound selected from the following compounds:

a) a nucleotide sequence according to the invention;

b) a polypeptide according to the invention;

c) a vector or a cell transformed according to the invention;

d) an antibody according to the invention; and

e) a compound capable of being selected by a selection method according to the invention, possibly in combination with a pharmaceutically acceptable vehicle.

Effective quantity is understood as designating a sufficient quantity of said compound or antibody, or of polypeptide of the invention, for modulating the cellular replication of PWD circovirus.

The invention likewise relates to a pharmaceutical composition according to the invention, for the prevention or the treatment of an infection by the PWD circovirus.

The invention is also directed at an immunogenic and/or vaccine composition, characterized in that it comprises one or more polypeptides according to the invention and/or one or more hybrid polypeptides according to the invention.

The invention also comprises the use of a cell transformed according to the invention, for the preparation of a vaccine composition.

The invention is likewise directed at a vaccine composition, characterized in that it contains a nucleotide sequence according to the invention, a vector according to the invention and/or a cell transformed according to the invention.

The invention likewise relates to the vaccine compositions according to the invention, for the prevention or treatment of an infection by the PWD circovirus.

The polypeptides of the invention entering into the immunogenic compositions according to the invention can be selected by techniques known to the person skilled in the art such as, for example, depending on the capacity of said polypeptides to stimulate the T cells, which is translated, for example, by their

proliferation or the secretion of interleukins, and which leads to the production of antibodies directed against said polypeptides.

In mice, in which a weight dose of the vaccine composition comparable to the dose used in man is administered, the antibody reaction is tested by taking of the serum followed by a study of the formation of a complex between the antibodies present in the serum and the antigen of the vaccine composition, according to the usual techniques.

According to the invention, said vaccine combinations will preferably be combined with a pharmaceutically acceptable vehicle and, if need be, with one or more adjuvants of the appropriate immunity.

Today, various types of vaccines are available for protecting animals or man against infectious diseases: attenuated living microorganisms (*M. bovis* - BCG for tuberculosis), inactivated microorganisms (influenza virus), acellular extracts (*Bordetella pertussis* for whooping cough), recombined proteins (surface antigen of the hepatitis B virus), polysaccharides (pneumococcal). Vaccines prepared from synthetic peptides or genetically modified microorganisms expressing heterologous antigens are in the course of experimentation. More recently still, recombined plasmid DNAs carrying genes coding for protective antigens have been proposed as an alternative vaccine strategy. This type of vaccination is carried out with a particular plasmid originating from a plasmid of *E.coli* which does not replicate *in vivo* and which codes uniquely for the vaccinating protein. Animals have been immunized by simply injecting the naked plasmid DNA into the muscle. This technique leads to the expression of the vaccine protein *in situ* and to an immune response of cellular type (CTL) and of humoral type (antibody). This double induction of the immune response is one of the

principal advantages of the vaccination technique with naked DNA.

The vaccine compositions comprising nucleotide sequences or vectors into which are inserted said sequences are especially described in the international application No. WO 90/11092 and likewise in the international application No. WO 95/11307.

The constitutive nucleotide sequence of the vaccine composition according to the invention can be injected into the host after having been coupled to compounds which favor the penetration of this polynucleotide into the interior of the cell or its transport to the cell nucleus. The resultant conjugates can be encapsulated in polymeric microparticles, as described in the international application No. WO 94/27238 (Medisorb Technologies International).

According to another embodiment of the vaccine composition according to the invention, the nucleotide sequence, preferably a DNA, is complexed with DEAE-dextran (Pagano et al., 1967) or with nuclear proteins (Kaneda et al., 1989), with lipids (Felgner et al., 1987) or encapsulated in liposomes (Fraley et al., 1980) or else introduced in the form of a gel facilitating its transfection into the cells (Midoux et al., 1993, Pastore et al., 1994). The polynucleotide or the vector according to the invention can also be in suspension in a buffer solution or be combined with liposomes.

Advantageously, such a vaccine will be prepared according to the technique described by Tacson et al. or Huygen et al. in 1996 or alternatively according to the technique described by Davis et al. in the international application No. WO 95/11307.

Such a vaccine can likewise be prepared in the form of a composition containing a vector according to the invention, placed under the control of regulation elements allowing its expression in man or animal. It will be possible, for example, to use, by way of *in*

vivo expression vector of the polypeptide antigen of interest, the plasmid pcDNA3 or the plasmid pcDNA1/neo, both marketed by Invitrogen (R&D Systems, Abingdon, United Kingdom). It is also possible to use the plasmid V1Jns.tPA, described by Shiver et al. in 1995. Such a vaccine will advantageously comprise, apart from the recombinant vector, a saline solution, for example a sodium chloride solution.

Pharmaceutically acceptable vehicle is understood as designating a compound or a combination of compounds entering into a pharmaceutical composition or vaccine which does not provoke secondary reactions and which allows, for example, the facilitation of the administration of the active compound, an increase in its duration of life and/or its efficacy in the body, an increase in its solubility in solution or alternatively an improvement in its conservation. These pharmaceutically acceptable vehicles are well known and will be adapted by the person skilled in the art as a function of the nature and of the mode of administration of the chosen active compound.

As far as the vaccine formulations are concerned, these can comprise adjuvants of the appropriate immunity which are known to the person skilled in the art, such as, for example, aluminum hydroxide, a representative of the family of muramyl peptides such as one of the peptide derivatives of N-acetyl muramyl, a bacterial lysate, or alternatively Freund's incomplete adjuvant.

These compounds can be administered by the systemic route, in particular by the intravenous route, by the intramuscular, intradermal or subcutaneous route, or by the oral route. In a more preferred manner, the vaccine composition comprising polypeptides according to the invention will be administered through the food or by nebulization several times, staggered over time.

Their administration modes, dosages and optimum pharmaceutical forms can be determined according to the criteria generally taken into account in the establishment of a treatment adapted to an animal such as, for example, the age or the weight, the seriousness of its general condition, the tolerance to the treatment and the secondary effects noted.

The present invention likewise relates to the use of nucleotide sequences of the PWD circovirus according to the invention for the construction of autoreplicative retroviral vectors and the therapeutic applications of these, especially in the field of human gene therapy in vivo.

The feasibility of gene therapy applied to man no longer needs to be demonstrated and this relates to numerous therapeutic applications like genetic diseases, infectious diseases and cancers. Numerous documents of the prior art describe the means of employing gene therapy, especially through viral vectors. Generally speaking, the vectors are obtained by deletion of at least some of the viral genes which are replaced by the genes of therapeutic interest. Such vectors can be propagated in a complementation line which supplies in trans the deleted viral functions in order to generate a defective viral vector particle for replication but capable of infecting a host cell. To date, the retroviral vectors are amongst the most widely used and their modes of infection are widely described in the literature accessible to the person skilled in the art.

The principle of gene therapy is to deliver a functional gene, called a gene of interest, of which the RNA or the corresponding protein will produce the desired biochemical effect in the targeted cells or tissues. On the one hand, the insertion of genes allows the prolonged expression of complex and unstable molecules such as RNAs or proteins which can be extremely difficult or even impossible to obtain or to

administer directly. On the other hand, the controlled insertion of the desired gene into the interior of targeted specific cells allows the expression product to be regulated in defined tissues. For this, it is necessary to be able to insert the desired therapeutic gene into the interior of chosen cells and thus to have available a method of insertion capable of specifically targeting the cells or the tissues chosen.

Among the methods of insertion of genes, such as, for example, microinjection, especially the injection of naked plasmid DNA (Derse, D. et al., 1995, and Zhao, T.M. et al., 1996), electroporation, homologous recombination, the use of viral particles, such as retroviruses, is widespread. However, applied in vivo, the gene transfer systems of recombinant retroviral type at the same time have a weak infectious power (insufficient concentration of viral particles) and a lack of specificity with regard to chosen target cells.

The production of cell-specific viral vectors, having a tissue-specific tropism, and whose gene of interest can be translated adequately by the target cells, is realizable, for example, by fusing a specific ligand of the target host cells to the N-terminal part of a surface protein of the envelope of the PWD circovirus. It is possible to mention, for example, the construction of retroviral particles having the CD4 molecule on the surface of the envelope so as to target the human cells infected by the HIV virus (YOUNG, J.A.T. et al., Sciences 1990, 250, 1421-1423), viral particles having a peptide hormone fused to an envelope protein to specifically infect the cells expressing the corresponding receptor (KASAHARA, N. et al., Sciences 1994, 266, 1373-1376) or else alternatively viral particles having a fused polypeptide capable of immobilizing on the receptor of the epidermal growth factor (EGF) (COSSET, F.L. et al., J. of Virology 1995, 69, 10, 6314-6322). In another approach, single-chain

fragments of antibodies directed against surface antigens of the target cells are inserted by fusion with the N-terminal part of the envelope protein (VALSESIA-WITTMAN, S. et al., J. of Virology 1996, 70, 3, 2059-2064; TEARINA CHU, T.H. et al., J. of Virology 1997, 71, 1, 720-725).

For the purposes of the present invention, a gene of interest in use in the invention can be obtained from a eukaryotic or prokaryotic organism or from a virus by any conventional technique. It is, preferably, capable of producing an expression product having a therapeutic effect and it can be a product homologous to the cell host or, alternatively, heterologous. In the scope of the present invention, a gene of interest can code for an (i) intracellular or (ii) membrane product present on the surface of the host cell or (iii) secreted outside the host cell. It can therefore comprise appropriate additional elements such as, for example, a sequence coding for a secretion signal. These signals are known to the person skilled in the art.

In accordance with the aims pursued by the present invention, a gene of interest can code for a protein corresponding to all or part of a native protein as found in nature. It can likewise be a chimeric protein, for example arising from the fusion of polypeptides of various origins or from a mutant having improved and/or modified biological properties. Such a mutant can be obtained, by conventional biological techniques, by substitution, deletion and/or addition of one or more amino acid residues.

It is very particularly preferred to employ a gene of therapeutic interest coding for an expression product capable of inhibiting or retarding the establishment and/or the development of a genetic or acquired disease. A vector according to the invention is in particular intended for the prevention or for the treatment of cystic fibrosis, of hemophilia A or B, of

Duchenne's or Becker's myopathy, of cancer, of AIDS and of other bacteria or infectious diseases due to a pathogenic organism: virus, bacteria, parasite or prion. The genes of interest utilizable in the present invention are those which code, for example, for the following proteins:

- a cytokine and especially an interleukin, an interferon, a tissue necrosis factor and a growth factor and especially a hematopoietic growth factor (G-CSF, GM-CSF),
- a factor or cofactor involved in clotting and especially factor VIII, von Willebrand's factor, antithrombin III, protein C, thrombin and hirudin,
- an enzyme or an enzyme inhibitor such as the inhibitors of viral proteases,
- an expression product of a suicide gene such as thymidine kinase of the HSV virus (herpesvirus) of type 1,
- an activator or an inhibitor of ion channels,
- a protein of which the absence, the modification or the deregulation of expression is responsible for a genetic disease, such as the CFTR protein, dystrophin or minidystrophin, insulin, ADA (adenosine diaminose), glucocerebrosidase and phenylhydroxylase,
- a protein capable of inhibiting the initiation or the progression of cancers, such as the expression products of tumor suppressor genes, for example the P53 and Rb genes,
- a protein capable of stimulating an immune or an antibody response, and
- a protein capable of inhibiting a viral infection or its development, for example the antigenic epitopes of the virus in question or altered variants of viral proteins capable of entering into competition with the native viral proteins.

The invention thus relates to the vectors characterized in that they comprise a nucleotide

sequence of PWD circovirus according to the invention, and in that they additionally comprise a gene of interest.

The present invention likewise relates to viral particles generated from said vector according to the invention. It additionally relates to methods for the preparation of viral particles according to the invention, characterized in that they employ a vector according to the invention, including viral pseudo-particles (VLP, virus-like particles).

The invention likewise relates to animal cells transfected by a vector according to the invention.

Likewise comprised in the invention are animal cells, especially mammalian, infected by a viral particle according to the invention.

The present invention likewise relates to a vector, a viral particle or a cell according to the invention, for the treatment and/or the prevention of a genetic disease or of an acquired disease such as cancer or an infectious disease. The invention is likewise directed at a pharmaceutical composition comprising, by way of therapeutic or prophylactic agent, a vector or a cell according to the invention, in combination with a vehicle acceptable from a pharmaceutical point of view.

Other characteristics and advantages of the invention appear in the examples and the following figures:

Legends to the figures:

Figure 1: Experimental scheme which has made it possible to bring about the circovirus associated with PWD.

Test 1: experimental reproduction of PWD by inoculation of pig organ homogenates from farms affected by PWD.

Test 2: experimental reproduction of PWD.

Test 3: experimental reproduction of PWD.

Test 4: no experimental reproduction of PWD.

Figure 2: Nucleotide sequence SEQ ID No. 1 of the genome of the circovirus associated with PWD, strand of (+) polarity.

Figure 3: Organization of the genome of the circovirus associated with PWD, strand of + and - polarity in the three reading frames.

Figure 4: Amino acid sequence of a polypeptide encoded by the nucleotide sequence ORF1 of the circovirus associated with PWD (strain FPW) corresponding to the REP protein.

Figure 5: Amino acid sequence of a polypeptide encoded by the nucleotide sequence ORF2 of the circovirus associated with PWD (strain FPW).

Figure 6: Amino acid sequence of a polypeptide encoded by the nucleotide sequence ORF3 of the circovirus associated with PWD (strain FPW).

Figure 7: Alignment of the nucleotide sequence of the PWD circovirus and of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.

Figure 8: Alignment of the sequence of amino acids of a polypeptide encoded by the nucleotide sequence ORF1 of the PWD circovirus and of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.

Figure 9: Alignment of the sequence of amino acids of a polypeptide encoded by the nucleotide sequence ORF2 of the PWD circovirus and of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.

Figure 10: Alignment of the sequence of amino acids of a polypeptide encoded by the nucleotide sequence ORF3

of the PWD circovirus and of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.

Figure 11: Western blot analysis of recombinant proteins of the PWD circovirus.

The analyses were carried out on cell extracts of Sf9 cells obtained after infection with recombinant baculovirus PCF ORF 1.

EXAMPLES

1 - Experimental procedures

Experimental reproduction of the infection and its syndrome (cf. Figure 1).

A first test was carried out with pigs from a very well-kept farm, but affected by piglet weight loss disease (PWD), likewise called fatal piglet wasting (FPW). Tests carried out with SPF (specific pathogen-free) pigs has [sic] showed a transfer of contaminant(s) finding expression in a complex pathology combining hyperthermia, retardation of growth, diarrhea and conjunctivitis. The PDRS (porcine dysgenic and respiratory syndrome) virus, an infectious disease due to an arteriovirus) was rapidly isolated from breeding pigs and contact pigs. It should have been possible to attribute all the clinical signs to the presence of the PDRS virus. However, two farm pigs presented signs of FPW without the PDRS virus being isolated. The histological analyses and blood formulas, however, showed that these pigs were suffering from an infectious process of viral origin.

In a second test, 8-week SPF pigs were inoculated by the intratracheal route with organ homogenates of two farm pigs suffering from FPW. The inoculated pigs exhibited hyperthermia 8 to 9 days post-infection, then their growth was retarded. Other SPF pigs, placed in contact, had similar, attenuated signs 30 days after the initial experiment. No

seroconversion with respect to a European or Canadian strain of PDRS virus was recorded in these animals.

A third test allowed the syndrome to be reproduced from samples taken from the pigs of the second test.

Conclusion

The syndrome is reproduced under the experimental conditions. It is determined by an infectious agent, which is transmittable by direct contact. The clinical constants are a sometimes high hyperthermia (greater than or equal to 41.5°C) which develops 8 to 10 days after infection. Retardation of the growth can be observed. The other signs are a reversal of the blood formula (reversal of the lymphocyte/polynuclear ratio from 70/30 to 30/70) and frequent lesions on the ganglia, especially those draining the respiratory apparatus (ganglionic hypertrophy, loss of structure with necrosis and infiltration by mononucleated or plurinucleated giant cells).

2 - Laboratory studies

Various cell supports including primary pig kidney cells or cell lines, pig testicle cells, monkey kidney cells, pig lymphocytes, pig alveolar macrophages and circulating blood monocytes were used to demonstrate the possible presence of a virus. No cytopathic effect was demonstrated in these cells. On the other hand, the use of a serum of a pig sick after experimental infection allowed an intracellular antigen to be revealed in the monocytes, the macrophages and approximately 10% of pig kidney cells infected with organ homogenates. This indirect revealing was carried out kinetically at different culture times. It is evident from this that the antigen initially appears in the nucleus of the infected cells before spreading into the cytoplasm. The successive passages in cell culture did not allow the signal to be amplified.

Under electron microscopy on organ homogenates, spherical particles labeled specifically by the serum of sick pigs, infected under the experimental conditions, were visualized. The size of these particles is estimated at 20 nm.

After two passages of these organ homogenates over pig lymphocytes and then three passages over pig kidney or testicle cells, a cytopathic effect developed and was amplified. An adenovirus was visualized in the electron microscope, which, under the experimental conditions, did not reproduce FPW (only a hyperthermia peak was noted 24 to 48 hours after infection, and then nothing more).

It has been possible to demonstrate DNA bands in certain samples of pigs infected under the experimental conditions and having exhibited signs of the disease (results not shown). A certain connection exists between the samples giving a positive result in cell culture and those having a DNA band.

Conclusion

Two viruses were demonstrated in the organ homogenates from pigs suffering from FPW. One is an adenovirus, but by itself alone it does not reproduce the disease. The other is a circovirus and is associated with FPW. This circovirus has been sequenced and it has mutations with respect to the known sequences on the circoviruses which are nonpathogenic for the pig.

3 - Cloning and sequencing of the DNA of the PWD circovirus

Extraction of the replicative form (RF) DNA, cleavage by the Kpn I enzyme and amplification by a pair of primers flanking the Kpn I restriction site. Sequencing of the two strands at least twice by the Sanger method.

4 - Comparison of the nucleotide sequences and amino acids of the PWD circovirus (or associated with PWD) which are obtained with

the corresponding sequences of MEEHAN and MANKERTZ circoviruses of porcine cell lines

Use of the DNA sequence analysis software, DNASIS.

Sequences of oligonucleotides used as primers or probes in the detection and/or identification procedures

1. specific detection of the PWD circovirus:

primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

primer PCV 10: 5' TGG AAT GTT AAC GAG CTG AG 3';

2. specific detection of the circovirus of the cell lines:

primer PCF 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

primer MEE 1: 5' TGG AAT GTT AAC TAC CTC AA 3';

3. differential detection:

the pairs of primers used are those described, for example, in the paragraphs 1 and 2 above;

4. detection of the monomeric circular replicative forms RF:

primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

primer PCV 6: 5' CTC GCA GCC ATC TTG GAA TG 3';

5. detection of the vectors carrying the dimers in tandem:

Nar dimer:

primer KS 620: 5' CGC GCG TAA TAC GAC TCA CT 3';

primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

Kpn dimer:

primer KS 620: 5' CGC GCG TAA TAC GAC TCA CT 3';

primer PCV 6: 5'CTC GCA GCC ATC TTG GAA TG 3';

6. differential detection:

the pairs of primers used are those described, for example, in paragraphs 4 and 5 above.

The procedures using the pairs or primers described in paragraphs 4 and 5 are of particular interest for differentially detecting the circular monomeric forms of specific replicative forms of the virion or of the DNA in replication and the dimeric forms found in the so-called in-tandem molecular constructs.

The in-tandem constructs of the viral genome (dimers) such as the constructs used for the preparation of the pBS KS + tandem PCV Kpn I vector, deposited at the CNCM under the number I-1891, 3 July 1197 [sic] (E. coli transformed by said vector) are very interesting for their use in methods of production in sufficient quantity of an inoculum formed of DNA, intended for the virus production, this in the absence of a satisfactory virus production protocol in a cell system. These said methods of production using these in-tandem constructs of the viral genome will allow the virulence factors to be studied by mutation and by way of consequence will be able to be used for the production of a collection of viruses carrying the mutations indicated in the construction of vectors which will have the appropriate tropism and virulence. These vectors with autoreplicative structure have the sought gene transfer properties, especially for their applications in gene therapy, and in vaccinology.

Western-blot analysis of recombinant proteins of the PWD circovirus

The results were obtained using a specific antiserum of the PWD circovirus produced during test 1 (cf. Figure 1).

Type of products analyzed.

The analyses were carried out on cell extracts of Sf9 cells obtained after infection by the recombinant baculovirus PCV ORF 1.

The culture of Sf9 cells was carried out in a 25 cm^2 Petri dish according to the standard culture methods for these cells. After centrifugation, the cell pellets are taken up with 300 μl of PBS buffer (phosphate saline buffer).

Electrophoresis (PAGE-SDS)

The electrophoresis is carried out on the cell extracts of Sf9 cells obtained previously on 5 samples (cf. Table 1 below) under the following conditions:
% polyacrylamide gel: 8%; conditions: denaturing

Voltage: 80 V; duration: 135 mn.

Table 1: Nature of the samples subjected to electrophoresis

Well No.	1	2	3	4	5
Sample applied	PM Rainbow	Raoul 24 h	Raoul 48 h	Raoul 72 h	Raoul 96 h
µl of sample	10	15	15	15	15
µl of Laemmli 4X	0	5	5	5	5

Legends to Table 1:

Laemmli 4X: loading buffer

PM Rainbow: molecular-weight markers (35, 52, 77, 107, 160 and 250 kD)

Raoul 24 h, 48 h, 72 h and 96 h: expression products of the ORF1 of the PWD circovirus.

Western blot

After electrophoresis, the bands obtained in the different wells are transferred to nitrocellulose membrane for 1 h at 100 v in a TGM buffer (tris-glycine-methanol).

The Western blot is carried out under the following conditions:

1) Saturation with a solution containing 5% of skimmed milk; 0.05% of Tween 20 in a TBS 1X buffer (tris buffer saline) for 30 min.

2) 1st antibody:

10 ml of PWD anticircovirus antibody are added diluted to 1/100, then the reaction mixture is incubated for one night at 4°C. Three washes of 10 min in TBS 1X are carried out.

3) 2nd antibody:

10 ml of pig rabbit P164 antibody anti-immunoglobulins, coupled to peroxidase (Dakopath) are added diluted to 1/100, then the reaction medium is

incubated for 3 hours at 37°C. Three washes of 10 min in TBS 1X are carried out.

4) Visualization

The substrate 4-chloro-1-naphthol in the presence of oxygenated water is used for visualization.

Results

The results are shown in Figure 11.

Kinetics of appearance of antibodies specific for the REP recombinant protein of the PWD circovirus expressed in baculovirus after infection of pigs by the PWD circovirus (test 4, cf. Figure 1)

After infections of the pigs, a sample of serum of each of the infected pigs is taken at different periods expressed in the table by the date of taking (carried out here in the same year) and is then analyzed by Western blot.

The visualization of the specific antibodies is carried out in the manner described previously.

The results obtained are shown by Table 2 below.

Table 2: Kinetics of appearance of specific antibodies

Sample	Pigs	10/6	16/06	23/06	01/07	08/07	15/07	21/07
A3	1						Neg.	
Control	2						Neg.	
B2	1	Neg.	Neg.	Neg.	+	+	++	+++
Infec.	2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
RP+	3	Neg.	Neg.	Neg.	Neg.	+	+	+
	4	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	++

Legends to Table 2:

A3 control: uninfected control animals;

B2 Infec. RP+: animals infected with pig kidney (PK) cells containing the circovirus;

Neg.: negative;

+, ++, +++: intensity scale of the positive reaction.

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